

# The Ability to Organize Sperm DNA into Functional Chromatin Is Acquired during Meiotic Maturation in Murine Oocytes

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Following fertilization of meiotically mature eggs, the chromatin of the sperm becomes biochemically and structurally remodeled within the egg cytoplasm. Despite the essential role of the paternal genome during embryogenesis, little is known of when the activities that regulate this chromatin remodeling appear during oogenesis. To determine whether these activities were acquired during meiotic maturation, we inseminated maturing oocytes of mice shortly after germinal vesicle breakdown. As previously shown, insemination at this stage did not activate the maturing oocytes, which became arrested at metaphase II. Immunofluorescent analysis revealed that at 1 hr postinsemination the sperm chromatin was dispersed and contained protamines but was devoid of core histones H2B and H3. At 4 hr postinsemination, both protamine and core histones were detectable on the sperm chromatin. By 8 hr postinsemination protamines were absent, and histones stained maximally. The appearance of immunoreactive histones was correlated with a morphological transition of the sperm chromatin from the dispersed to a condensed state, which suggests that the assembly of the histones reflected modification of the chromatin to a somatic-like state in which it was competent to respond to the metaphase-promoting factor activity of the oocyte. Both the assembly of histones and chromatin condensation were reversibly blocked when protein synthesis was inhibited, indicating that the remodeling process required proteins synthesized during maturation. Injection of core histones into protein synthesis-inhibited oocytes failed to induce condensation of the sperm chromatin, which implies that correct remodeling requires synthesis during maturation of nonhistone proteins. To test the functional capacity of remodeled sperm chromatin, maturing oocytes were inseminated, allowed to continue maturation for 17 hr and then parthenogenetically activated. Following activation, the sperm-derived chromatin as well as that of the oocyte became decondensed within pronuclei and underwent DNA replication, indicating that sperm chromatin remodeled in maturing oocyte cytoplasm was functionally normal. When the postinsemination incubation time was reduced to 11 hr; however, neither the female nor the male pronuclei underwent DNA replication, implying that factors synthesized late during maturation are required for DNA replication after activation. Taken together, these results indicate that the ability to organize sperm DNA into functional somatic-like chromatin develops in oocytes during meiotic maturation, requires proteins synthesized during maturation, and can be expressed independently of activation. © 1997 Academic Press

## INTRODUCTION

During oogenesis, the oocyte progressively acquires the ability to undergo normal fertilization and embryonic development. In many nonmammalian organisms, certain mRNAs and proteins accumulate in specific regions of the developing oocyte, and this localization is crucial for generation of the embryonic axes and differentiation of specific cell types (reviewed in Lasko, 1992; St Johnston, 1995). In mammals, numerous morphological, ultrastructural, and biochemical changes occur during oogenesis that are required for subsequent development. It is well-established,

for example, that at a specific stage of growth oocytes acquire the ability to undergo meiotic maturation and that this is correlated with an increase in the quantity of p34<sup>cdc2</sup> (Chesnel and Eppig, 1995; de Vantéry *et al.*, 1996; Mitra and Schultz, 1996) and with changes in microtubular average length and the appearance of phosphorylated epitopes in the microtubule-organizing centers (Wickramasinghe and Albertini, 1992). Further growth beyond this stage of meiotic competence is required for oocytes to acquire the capacity to develop to the blastocyst stage following fertilization (Eppig and Schroeder, 1989). This likely reflects the synthesis in the growing oocyte of mRNAs and proteins that are

required during early embryogenesis; for example, the actin-associated protein, ezrin, is synthesized by the oocyte and in morula-stage embryos becomes localized to the apical surface of the outer cells (Louvet *et al.*, 1996).

Many of the events that prepare the oocyte for the initiation of embryonic development occur during meiotic maturation. The ability of the cortical granules to undergo exocytosis increases during maturation (Ducibella *et al.*, 1990; Ducibella and Buetow, 1994) and this is accompanied in some species by a migration from subcortical to cortical regions (Cran and Cheng, 1985). Clusters of endoplasmic reticulum also develop in the oocyte cortex during maturation, possibly due to migration from the deeper cytoplasm (Mehlmann *et al.*, 1995). Studies from several laboratories have established that the amount of calcium released by the oocyte in response to sperm and chemical calcium-releasing agents increases during maturation (Tombes *et al.*, 1992; Fujiwara *et al.*, 1993; Mehlmann and Kline, 1994; Jones *et al.*, 1995) and that the dynamics of this calcium wave also change (Carroll *et al.*, 1994). All of these changes probably contribute to the acquisition late during maturation by the oocyte of the ability to be activated and enter the mitotic cell cycle in response to sperm penetration or parthenogenetic stimuli (Kubiak, 1989).

Among the earliest events of embryonic development is the biochemical and structural reorganization of the sperm chromatin within the oocyte cytoplasm. Protamines or sperm-specific histones are removed, egg histones and other proteins are assembled onto the sperm DNA, and this becomes packaged into nucleosomal chromatin. The sperm-derived chromatin then becomes decondensed within the paternal pronucleus and DNA replication is initiated. This reorganization must proceed rapidly and accurately to ensure that the entire paternal haploid genome is transmitted to the embryo. Additionally, in mammals, the paternal genomic imprinting pattern must be retained or established during this remodeling process (Sapienza *et al.*, 1987). As the sperm contains essentially no nucleoplasm or cytoplasm, the reorganization of its chromatin must be effected by activities present within the oocyte.

Despite their essential role in the assembly of functional paternal chromatin, however, almost nothing is known of when the activities appear during oocyte development. When oocytes are inseminated at a late stage of meiotic maturation, the sperm form pronuclei, whereas when they are inseminated at earlier stages, the sperm do not form pronuclei (Usui and Yanagimachi, 1976; Zirkin *et al.*, 1989). It is not clear whether this result reflects the ability of the oocyte to remodel the sperm chromatin or of the oocyte to be activated, as chromatin decondensation and nuclear formation would be expected to occur only in an interphase cytoplasm, like that found in an activated egg. Other experiments have examined the fate of sperm chromatin within partially mature oocytes that are not activated by sperm penetration. Within the first few hours of residence within the oocyte cytoplasm, the sperm nuclear envelope is removed and the chromatin becomes dispersed (Szollosi *et*

*al.*, 1990). These initial changes do not occur in oocytes that are maintained in prophase arrest. Later, the dispersed chromatin becomes condensed into a small mass (Iwamatsu and Chang, 1972) or into chromosome-like structures (Clarke and Masui, 1986). These become associated with microtubules that frequently form spindles (Harrouk and Clarke, 1993).

These results suggest that the maturing oocyte has a capacity to alter the morphology of the sperm chromatin. But, as neither the biochemical composition nor the functional capacity of this sperm-derived chromatin have been examined, it is not known whether the morphological change reflects the assembly of physiologically normal chromatin. To test whether the ability to organize the sperm into somatic-like chromatin is present or acquired during oocyte maturation, we examined whether maturing oocyte cytoplasm could mediate the removal of sperm protamines followed by the addition of egg histones and whether sperm chromatin that had been remodeled in the oocyte cytoplasm could subsequently form a pronucleus and undergo DNA replication.

## MATERIALS AND METHODS

### *Collection and Fertilization of Oocytes*

Oocytes containing a germinal vesicle (GV) were collected from ovarian follicles of 21-day CD-1 female mice (Charles River Canada) and incubated in 5- $\mu$ L microdrops of bicarbonate-buffered minimal essential medium (MEM; Life Technologies, Grand Island, NY) under oil at 37°C in an atmosphere of 5% CO<sub>2</sub> in air, hereafter designated the standard conditions. After 3 hr, those oocytes that had undergone germinal vesicle breakdown (GVBD) were exposed to acidified (pH 2.5) Tyrode's medium to remove the zona pellucida and then transferred to a well containing 450  $\mu$ L of IVF-MEM (MEM containing 4 mg/mL bovine serum albumin freshly added).

One epididymis from each of two 13-week CD-1 or B2F6F1 hybrid males (Charles River Canada) was removed, transferred to a dish containing 500  $\mu$ L of IVF-MEM, and punctured to release mature sperm. The dish was incubated under the standard conditions for 20 min, and then the epididymi were removed and the remaining suspension was returned to the incubator and allowed to capacitate for an additional 1 hr 40 min.

Sperm was added to the oocyte-containing well at a concentration of  $2 \times 10^5$  mL<sup>-1</sup>, and insemination was allowed to occur for 1 hr. After insemination, sperm adhering to the surface of the oocyte were removed by gentle aspiration in a micropipette. Oocytes were incubated in MEM microdrops under the standard conditions for between 2 and 24 hr, and then manipulated according to the required procedure.

### *Parthenogenetic Activation*

After insemination, oocytes were incubated overnight in MEM microdrops. Parthenogenetic activation was achieved by a 3.5-min exposure to 8% ethanol in Hepes-buffered MEM (MEM-H), three washes in fresh MEM-H, then incubation for 9 hr in 100  $\mu$ g/mL puromycin (Sigma Chemical Co., St. Louis, MO) in MEM microdrops. Following the puromycin incubation, cells were fixed in 10%

formalin (Sigma) for 10 min and permeabilized for 2.5 min in 0.1% Triton-X (Sigma) in phosphate-buffered saline (PBS). Oocytes were mounted in Moviol (Hoechst Canada Inc., Montreal, QC) containing 1 mg/mL 4',6-diamidino-2-phenylindolidehydrochloride (DAPI; Boehringer-Mannheim Canada, Laval, QC). Activated oocytes containing three or more pronuclei were scored as having been penetrated by a sperm. As a test of this scoring method, the following trial was performed. Inseminated oocytes were divided into two groups. One group was fixed and the penetration rate calculated by counting the number of oocytes that contained remodeled sperm chromatin. The other group was activated and then scored for penetration by observing the number of pronuclei. In all cases (five trials,  $n = 302$ ) the penetration rate determined following activation was equal to or lower than that determined for the unactivated group. This supports the argument that the activated oocytes which contained three or more pronuclei had been penetrated by sperm.

### Labeling of Newly Synthesized DNA

After insemination, oocytes were incubated under the standard conditions for 11 or 17 hr and then parthenogenetically activated as above. Following activation oocytes were incubated under standard conditions overnight in 0.4 mM 5'-bromo-2-deoxyuridine (BrdU, Boehringer-Mannheim) in MEM. Oocytes were fixed and processed for immunocytochemistry.

### Immunocytochemistry

Immunocytochemistry was performed as previously described (see Clarke *et al.*, 1992). Core histones were detected using affinity-purified anti-H2B and anti-H3 primary antibodies prepared and characterized as described (Bustin, 1989; kindly supplied by Dr. M. Bustin; dilution, 1:50). Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (BioCan Scientific, Mississauga, ON; dilution, 1:100) was used as the secondary antibody. For protamine detection, a mouse monoclonal anti-human 1N primary antibody known to cross-react with mouse protamine (Stanker *et al.*, 1987; kindly supplied by Dr. R. Balhorn; dilution, 1:10) and a FITC-conjugated anti-mouse antibody were used. For BrdU detection, oocytes were incubated in undiluted mouse anti-BrdU primary antibody (Boehringer Mannheim) together with 100  $\mu$ g/mL DNase, washed, and then incubated in a FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories; dilution, 1:100). Stained oocytes were mounted in Moviol with 1  $\mu$ g/mL DAPI and viewed with a Leitz Laborlux S microscope with a UV attachment. In Fig. 4, H2B staining has been digitally enhanced. For protamine detection, slides were viewed using a Leica Confocal Laser Scanning Microscopy system.

### Inhibition of Protein Synthesis

For protein synthesis inhibition trials, inseminated oocytes were incubated in puromycin for 2, 4, 6, or 8 hr and then transferred to puromycin-free MEM and incubated either for an additional 6 hr in the 2-hr puromycin trial or for 8 hr in all other trials. In some cases (see Results), oocytes were incubated in puromycin-free MEM up to 22 or 24 hr after insemination. Inhibition of protein synthesis by puromycin was confirmed by the inability of puromycin-treated 1-cell embryos to cleave to the 2-cell stage (not shown).

### Microinjection of Histone

Oocytes containing a GV were collected and incubated under standard conditions in MEM supplemented with 0.5 mg/mL dibutyryl cyclic AMP (Boehringer-Mannheim) to inhibit GVBD. Approximately 10 pL either of rhodamine-labeled core histones prepared as described (Minden *et al.*, 1989; Lin and Clarke, 1996) or of 1 mg/mL core histone (Boehringer-Mannheim) in PBS was microinjected into the cytoplasm of GV-containing oocytes using a Leitz Labovet FS microscope with Leitz M-type micromanipulators (Leica Canada, Montreal, QC). Injected oocytes were transferred to MEM microdrops and incubated for 3 hr before insemination to allow meiotic maturation to begin. Following insemination, oocytes were incubated in 100  $\mu$ g/mL puromycin for 8 hr before fixation and mounting. Those oocytes injected with 1 mg/mL histone were stained using the anti-H2B antibody before mounting.

## RESULTS

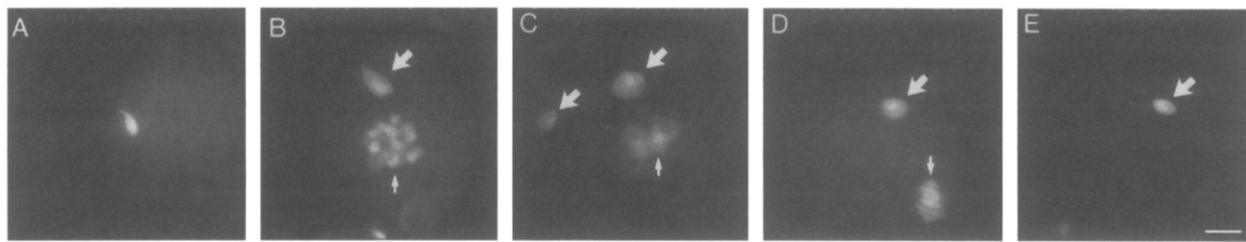
### Morphological Remodeling of Sperm Chromatin

When oocytes that have undergone GVBD are freed of the zona pellucida and then inseminated, sperm are able to penetrate into the oocyte cytoplasm where they undergo a characteristic sequence of morphological changes (Fig. 1). Initially, the chromatin within the intact sperm head disperses, reaching a maximum volume by 1 hr after insemination. During this dispersion, the intensity of the DAPI staining decreases while remaining uniform over the sperm chromatin. By 8 hr after insemination, the sperm chromatin becomes recondensed, such that it appears as a small, more intensely staining sphere that is clearly distinct from the hooked shape of an intact sperm head. During this period, the oocyte chromosomes become organized on a spindle, undergo the first meiotic division, and become arrested at metaphase II.

These morphological changes in sperm chromatin are the same as those that occur after fertilization at metaphase II (Wright and Longo, 1988). However, whereas the dispersion and recondensation occur within approximately 2 hr after normal fertilization of metaphase II eggs (Wright and Longo, 1988), our results showed that these processes required 8 hr in a meiotically maturing oocyte. When sperm penetration has occurred at metaphase II, this recondensed chromatin sphere subsequently decondenses to form the male pronucleus. When sperm penetration has occurred shortly after GVBD, the recondensed mass often resolves into metaphase-like chromosomes (Figs. 3C and 3D; Clarke and Masui, 1986). In both cases, however, the ultimate morphological appearance of the sperm chromatin matches that of the oocyte chromatin.

### Replacement of Protamines by Core Histones during Chromatin Remodeling

These results indicate that maturing oocyte cytoplasm can induce morphological changes to sperm chromatin similar to those induced by cytoplasm of fully mature oocytes.



**FIG. 1.** Sperm chromatin undergoes morphological changes following insemination during meiotic maturation of oocyte. (A) Mature sperm head. (B) 1 hr postinsemination (h.p.i.), sperm chromatin (large arrow) is maximally dispersed. (C) 4 h.p.i. Sperm chromatin remains dispersed. Oocyte is dispermic. (D) 6 h.p.i., recondensation of sperm chromatin is evident. (E) 8 h.p.i., sperm chromatin is maximally recondensed. Oocyte chromatin (small arrow) is condensed into meiotic bivalents in (B), out of the plane of focus in (C), and arranged on metaphase plate in (D). Samples stained with DAPI. Bar, 10  $\mu$ m.

To examine whether similar biochemical changes were also effected, we examined whether protamines were removed and histones assembled onto the sperm chromatin within the maturing oocyte cytoplasm.

Oocytes were allowed to undergo GVBD, inseminated, and then incubated for 1, 4, or 8 hr. The oocytes were then fixed and stained using an antibody against human protamine 1N, which cross-reacts with mouse protamines (Stanker *et al.*, 1987). Figure 2 shows the change in protamine staining of sperm chromatin during residence in oocyte cytoplasm. Strong anti-protamine staining was evident at 1 hr ( $n = 34$ ) and weaker staining at 4 hr ( $n = 12$ ) postinsemination. Note that the oocyte chromosomes in Fig. 2A were not stained by the antibody. In contrast, no staining was detectable on sperm chromatin incubated for 8 hr ( $n = 56$ ) in oocyte cytoplasm. Thus, protamines become undetectable on the sperm chromatin after between 4 and 8 hr residence in maturing oocyte cytoplasm.

After fertilization at metaphase II, the loss of protamines from the sperm chromatin is accompanied by the appearance of core histones (Ecklund and Levine, 1975; Nonchev and Tsanev, 1990). Since the sperm chromatin lost detectable protamines within the cytoplasm of the maturing oocyte, we next analyzed whether it acquired core histones H2B and H3. Oocytes were allowed to undergo GVBD, inseminated, incubated overnight to allow meiosis to progress to metaphase II, and then processed for immunofluorescence using antibodies raised against histones H2B and H3 (Bustin, 1989). Both species of histone were detectable on the maternal chromosomes and also on sperm chromatin that had undergone the morphological remodeling process (Fig. 3). In contrast, sperm that had not penetrated the oocyte and remained morphologically unchanged stained negatively for H2B and H3. The novel appearance on the remodeled sperm chromatin of histones H2B and H3 indicates that its biochemical composition changed during residence in the maturing oocyte cytoplasm to contain at least these two core histones.

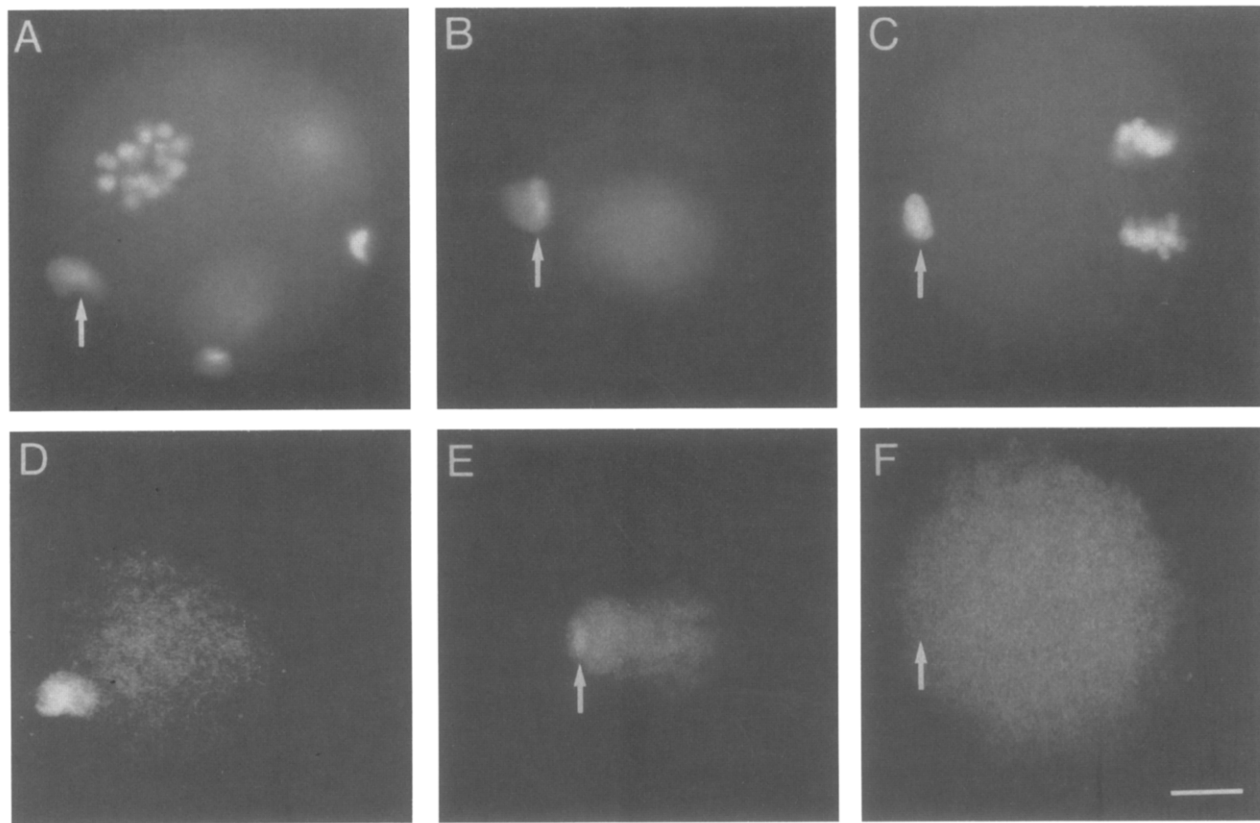
To define when histones first appeared during the remodeling process, we performed a time course analysis using histone H2B. Oocytes were allowed to undergo GVBD, in-

seminated, and then fixed at 2, 4, 6, 8, and 24 hr after the end of insemination. At 2 hr postinsemination the sperm chromatin had become dispersed, but did not contain detectable histone H2B. By 4 and 6 hr postinsemination the chromatin had become partially recondensed and in some cases stained positively for histone H2B, whereas in other cases no histone H2B staining was detectable. By 8 hr and also at 24 hr postinsemination, the chromatin was recondensed and maximally stained for histone H2B (Fig. 4, Table 1). Thus, histone H2B became detectable on the remodeling chromatin between 4 and 8 hr after penetration. In addition the appearance of histone H2B was correlated with the transition from dispersed to condensed chromatin state.

### **Dependence of Core Histone Assembly and Chromatin Remodeling on Protein Synthesis**

As noted above, the dispersion and recondensation of sperm chromatin seen within oocytes during meiotic maturation are similar to the early morphological changes displayed by mammalian sperm chromatin after normal fertilization at metaphase II but occur more slowly. One possible explanation for this difference is that the remodeling process requires a component which is synthesized during meiotic maturation, and that the progression of remodeling is dependent on synthesis of this component. To test this idea, we blocked protein synthesis and then observed the ability of the meiotically maturing oocyte to remodel sperm chromatin.

Oocytes were incubated for 3 hr to allow them to undergo GVBD and then inseminated and incubated for an additional 8 h in the presence or absence of puromycin. Those incubated in puromycin-free medium were recondensed and stained positively for H2B by 8 h after insemination. In contrast, of the 112 oocytes incubated for 8 hr in the presence of puromycin, 105 contained sperm chromatin arrested in a maximally dispersed state. Furthermore, the sperm chromatin did not contain detectable histone H2B. The remaining oocytes contained dispersed sperm chromatin that stained faintly for H2B. In a separate trial, oocytes assayed for the presence of protamines ( $n = 8$ ) showed no detectable



**FIG. 2.** Protamines become undetectable on sperm chromatin (arrows) during remodeling process. Oocytes were inseminated following GVBD and fixed at (A, D) 1 hr postinsemination (h.p.i.), (B, E) 4 h.p.i., and (C, F) 8 h.p.i. (A, B, C) DAPI-staining. (A) Dispersed sperm chromatin is indicated by arrow. Condensed oocyte chromosome bivalents are present in the upper left quadrant. Two in-focus and two out-of-focus nuclei representing sperm that failed to penetrate the oocyte are visible on the right side of the micrograph. (B) Dispersed sperm chromatin is indicated by arrow. Condensed oocyte chromosomes are out of the focal plane. (C) Condensed sperm chromatin is indicated by arrow. Oocyte chromosomes are at anaphase I. (D, E, F) staining with anti-protamine primary and FITC-conjugated secondary antibodies, viewed with confocal microscopy. Protamines are present strongly at 1 h.p.i., weakly at 4 h.p.i., and absent at 8 h.p.i. Increased sensitivity in (E, F) used to highlight decrease in antibody staining also increased slightly nonspecific cytoplasmic signal. Bar, 10  $\mu$ m.

staining on the remodeling chromatin (not shown). Thus, in the presence of the protein synthesis inhibitor, neither the recondensation of the sperm chromatin nor the appearance of core histone H2B could occur. This requirement for protein synthesis during meiotic maturation differs from the ability of eggs fertilized at metaphase II, which are able to remodel the sperm chromatin into the male pronucleus in the absence of protein synthesis (Wright and Longo, 1988).

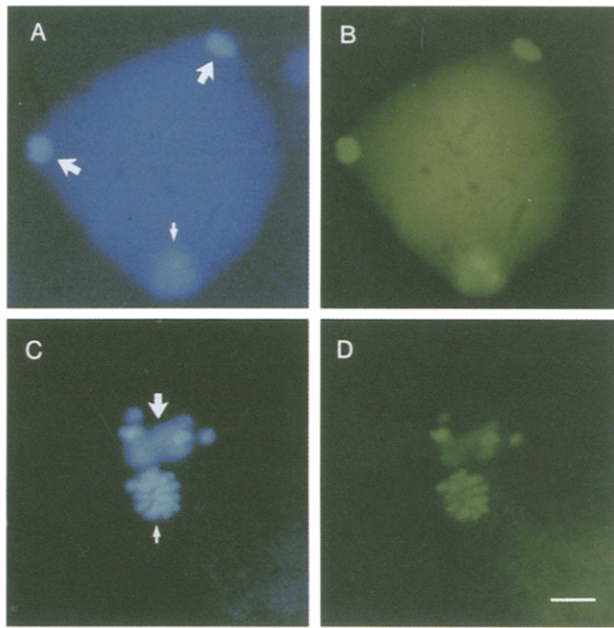
To test whether the effect of protein synthesis inhibition was reversible, inseminated oocytes were incubated in the presence of puromycin for 2, 4, 6, or 8 hr, transferred to puromycin-free medium for 6 hr, after the 2-hr puromycin trial and 8 hr after all other groups, and then fixed. As shown in Table 2, in all groups the sperm chromatin had become recondensed and was stained positively for H2B. The degree of recondensation was less in those groups incubated in the presence of puromycin for 6 or 8 hr than those incubated

for 2 or 4 hr, suggesting that recovery may have been delayed following longer puromycin treatment. However, the sperm chromatin became recondensed and H2B-positive in all cases when the puromycin-treated oocytes were incubated for 22 hr in puromycin-free medium (data not shown). Thus, it appears that the chromatin remodeling process can recover from a block to protein synthesis of at least 8 hr.

#### ***Inability of Microinjected Histones to Induce Sperm Chromatin Remodeling***

These results showed that, when protein synthesis was inhibited, sperm chromatin remained decondensed and also failed to stain positively for histone H2B. We therefore considered the possibility that histones synthesized during meiotic maturation might be the proteins necessary for remodeling of the sperm chromatin to the condensed state. To test this idea, we investigated whether exogenously sup-





**FIG. 3.** Core histones H2B and H3 are present on remodeled sperm chromatin. (A, C) DAPI-stained sperm chromatin (large arrows) remodeled in maturing oocyte for 18 hr. Oocyte chromosomes are arrested at metaphase II (small arrows). (B) Anti-H2B and (D) anti-H3 primary antibodies and FITC-conjugated secondary antibodies stain remodeled sperm chromatin. Position of sperm chromatin at periphery of oocyte shown in (A) and (B) is an artifact of fixation. This oocyte is dispermic. Sperm chromatin in the oocyte shown in (C) and (D) has resolved into metaphase-like chromosomes. Bar, 10  $\mu$ m.

plied core histones could overcome the protein synthesis requirement.

In the first series of experiments, GV-stage oocytes were injected with a preparation of histones that had been labeled with rhodamine (Minden *et al.*, 1989; Lin and Clarke, 1996). The injected oocytes were then inseminated and incubated in the presence of puromycin for 8 hr. When these oocytes were fixed and examined, the sperm chromatin was found to be in the maximally dispersed state ( $n = 59$ ). Rhodamine fluorescence was associated with the sperm chromatin (not shown), but the signal was of variable intensity over the chromatin, small foci of intense staining being present within a fainter background. These variations in the rhodamine fluorescence were not reflected in the DAPI staining pattern, which was uniform over the sperm chromatin. Fluorescence was also associated with the maternal chromatin, and with small cortex-associated foci that did not stain with DAPI.

In the second series, approximately 10 pg of unlabeled histones, which represents about two haploid-equivalents, was injected after which oocytes were inseminated and incubated for 8 hr in the presence of puromycin. Following this incubation, histone H2B was detectable on the dis-

persed chromatin and, in contrast to the results obtained using rhodamine-labeled histones, was apparently uniformly distributed over the sperm chromatin (Fig. 5). However, despite the presence of histone on the sperm chromatin, it failed to progress beyond the maximally dispersed state ( $n = 30$ ). Control experiments in which oocytes were microinjected, inseminated, and incubated in puromycin-free medium revealed that microinjection did not negatively affect the oocyte's ability to fully remodel sperm chromatin ( $n = 18$ ). Together, these results indicate that, although injected core histones are able to associate with the remodeling chromatin, their presence is not sufficient to induce the transition from dispersed to recondensed chromatin in the absence of protein synthesis.

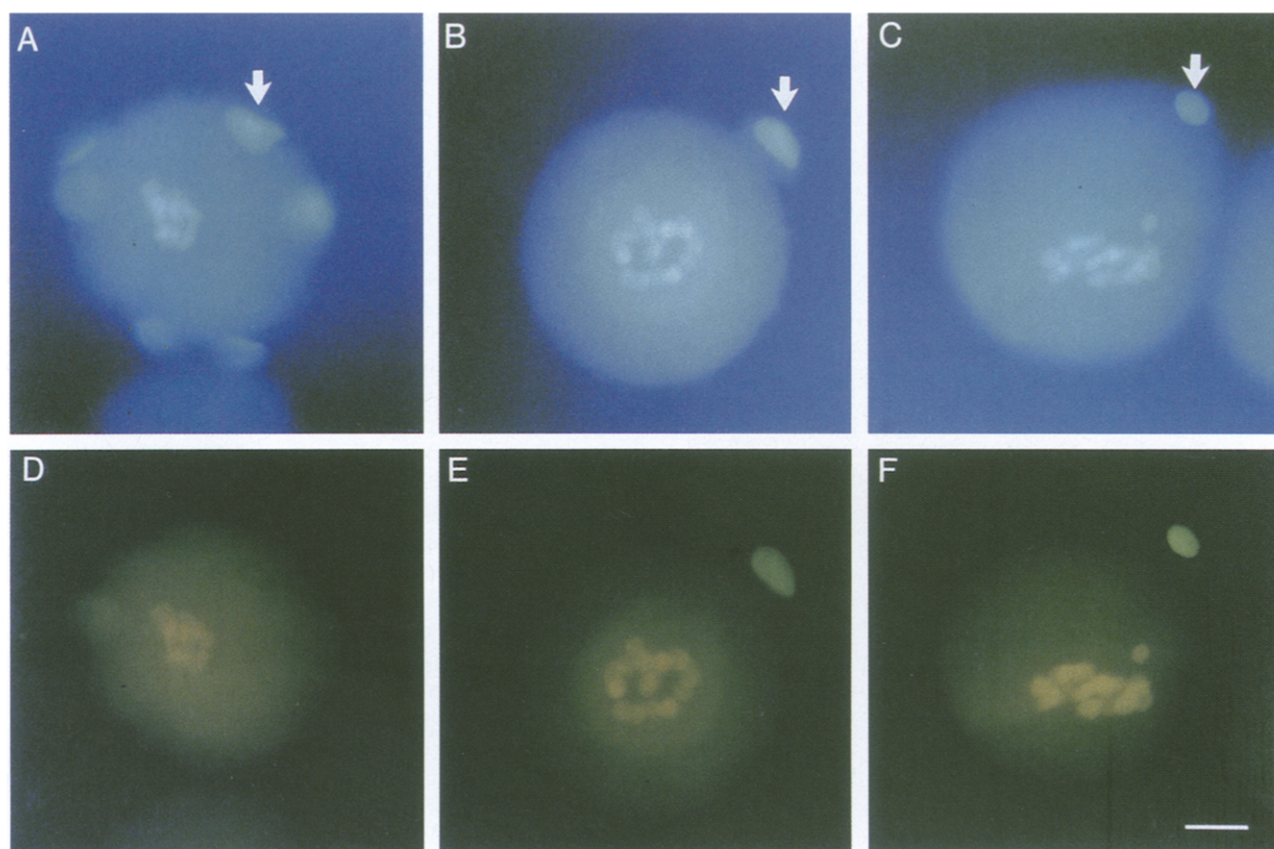
### **Formation of a Pronucleus from the Remodeled Sperm Chromatin Following Oocyte Activation**

The results described above demonstrate that the cytoplasm of the maturing oocyte is able to induce morphological and biochemical changes in sperm chromatin that are similar to those that occur within the cytoplasm of the fully mature egg. To test whether sperm that had been remodeled in maturing oocyte cytoplasm also possessed the same functional properties as those that are acquired after fertilization at metaphase II, we investigated whether such remodeled chromatin could form a male pronucleus and support DNA replication.

Oocytes were inseminated during maturation and incubated until 24 hr had elapsed from the beginning of maturation. They were then treated with ethanol and puromycin to induce parthenogenetic activation and then immediately fixed and examined. As is shown in Fig. 6, activation of these oocytes resulted in the maternal chromosomes completing meiosis, though not extruding a second polar body, thus forming two pronuclei. The remodeled male chromatin formed a distinct pronucleus that was morphologically similar to the female pronuclei. In all cases ( $n = 28$ ), activation resulted in formation of two female pronuclei and a male pronucleus. The pronuclei were distinguished by size, the male being larger, and location, the female pronuclei being adjacent to each other and the male pronucleus a greater distance away.

### **DNA Synthesis in the Sperm-Derived Pronucleus**

To test whether the remodeled sperm chromatin could undergo DNA replication, inseminated oocytes were parthenogenetically activated as above, incubated overnight (18 hr) in medium containing 0.4 mM BrdU, and then fixed and stained using an anti-BrdU antibody. In the first trials, the inseminated oocytes were incubated for 11 hr before parthenogenetic activation. Although both male and female pronuclei formed within the activated oocytes, no incorporation of BrdU could be detected in any of the pronuclei (Table 3). In the second trials, therefore, the period of incubation between insemination and parthenogenetic activa-



**FIG. 4.** Core histone H2B appears on sperm chromatin (arrows) during the remodeling process. Oocytes were inseminated following GVBD and fixed at (A, D) 2 hr postinsemination (h.p.i.), (B, E) 4 h.p.i., and (C, F) 8 h.p.i. (A, B, C) DAPI staining. (D, E, F) Staining with anti-H2B primary and FITC-conjugated secondary antibodies. Histone H2B staining first appears at 4 h.p.i., coincident with recondensation of sperm chromatin. Staining is maximal by 8 h.p.i. Position of sperm chromatin at periphery of oocyte is an artifact of fixation. Bar, 10  $\mu$ m.

tion was extended to 17 hr. In this case, BrdU was incorporated into all female pronuclei and 78% of the male pronuclei in the activated oocytes. These results indicate that sperm chromatin that has undergone morphological and biochemical remodeling in maturing oocyte cytoplasm is competent to replicate DNA, and suggest further that the ability of oocyte cytoplasm to support DNA replication may be acquired during the latter stages of maturation *in vitro*.

## DISCUSSION

### *Protamine-Histone Exchange on Sperm Chromatin within Maturing Oocytes*

We have investigated the ability of the meiotically maturing oocyte to remodel the nucleus of the sperm into somatic

chromatin. Our results show that, within the cytoplasm of maturing oocytes, protamines are removed and core histones H2B and H3 are assembled onto sperm chromatin,

**TABLE 1**

Time Course of Appearance of Histone H2B on Sperm Chromatin Following Insemination during Oocyte Maturation

Duration of postinsemination incubation (hr)	Number of eggs examined	H2B staining <sup>a</sup>		
		—	+/-	+
2	16	16	0	0
4	15	5	10	0
6	9	0	2	7
8	13	0	0	13

<sup>a</sup> Inseminated oocytes were fixed and stained with anti-H2B at 2, 4, 6, and 8 hr postinsemination.

**TABLE 2**  
Remodeling Process Recovery from Protein Synthesis Inhibition

Duration of puromycin incubation (hr)	Duration of post-puromycin incubation (hr)	Number of eggs examined	H2B staining <sup>a</sup>	
			-	+
2	6	25	1	24
4	8	18	5	13
6	8	15	11	4
8	8	20	3	17

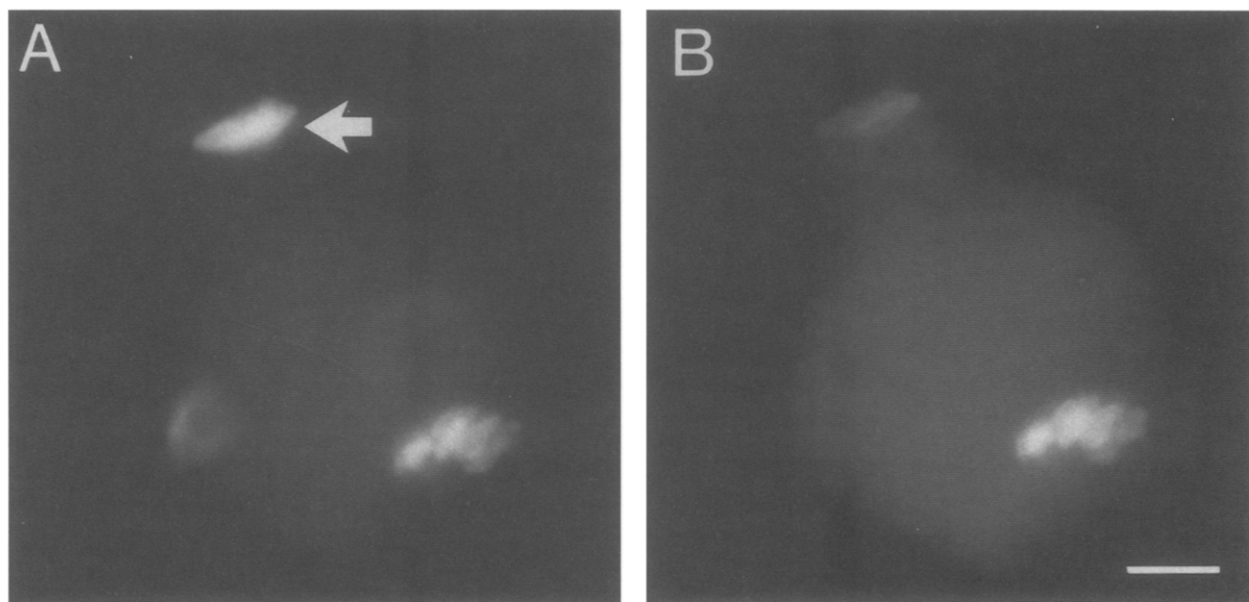
<sup>a</sup> Inseminated, maturing oocytes were incubated in MEM with 100  $\mu\text{g}/\text{mL}$  puromycin for 2, 4, 6, or 8 hr, transferred to puromycin-free MEM for 6 or 8 hr, fixed, and processed for the presence of H2B.

reproducing the transition that normally occurs following fertilization at metaphase II. Protamines were present at 1 hr after insemination when histones were undetectable, weakly detected or absent at 4 hr when the histones could first be detected, and entirely absent by 8 hr, when the histones were abundant on the sperm chromatin. The fact that loss of protamines was temporally correlated with assembly of histones suggests these processes were coordinated by factors within the oocyte cytoplasm. As the nucleoprotein switch had begun by 4 hr after insemination, which corresponds to 8 hr after the start of maturation and

precedes first polar body formation, it appears that the oocyte factors able to mediate the protamine removal and histone addition are present and active before metaphase I of maturation.

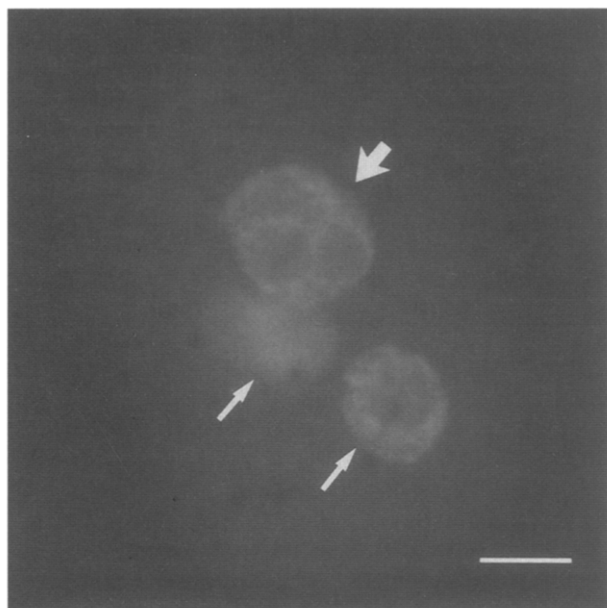
There was a strong correlation between the assembly of histones H2B and H3 onto the sperm chromatin and its morphological appearance, the histones being undetectable on dispersed chromatin and present on chromatin that had undergone the transition to form a condensed mass. Chromosome condensation at metaphase is regulated by metaphase-promoting factor (MPF), and it may be proposed that the condensation of the histone-containing sperm chromatin is due to the high MPF activity in the maturing oocyte. In contrast, the decondensation of the sperm chromatin within the male pronucleus following fertilization at metaphase II is due to the absence of MPF (Weber *et al.*, 1991; Kubiak *et al.*, 1992). We propose that the assembly of histones we have observed reflects a widespread structural and biochemical modification of the sperm chromatin within the maturing oocyte, which as a result is able to respond to its cytoplasmic environment.

Both assembly of core histones onto sperm chromatin and morphological transition to the condensed state required protein synthesis. Both of these processes occurred before metaphase I in untreated inseminated oocytes, which implies that the necessary proteins are synthesized during the early stages of maturation. As histones are known to be synthesized by oocytes (Wassarman and Letourneau, 1976; Wassarman and Mrozak, 1981), we tested whether microin-



**FIG. 5.** Microinjection of core histones does not allow remodeling in the absence of protein synthesis. Oocytes were microinjected with 10 pg core histones, inseminated, incubated in 100  $\mu\text{g}/\text{mL}$  in MEM for 8 hr, and then fixed. (A) DAPI staining of sperm chromatin (arrow) arrested in maximally decondensed state. (B) Staining with anti-H2B primary and FITC-conjugated secondary antibodies showing presence of histone on decondensed sperm chromatin. Bar, 10  $\mu\text{m}$ .





**FIG. 6.** Remodeled sperm chromatin can form a pronucleus upon parthenogenetic activation and synthesize DNA. Inseminated oocytes were parthenogenetically activated then incubated 18 hr in 0.4 mM BrdU. Oocytes were then fixed and stained with DAPI and anti-BrdU primary and FITC-conjugated secondary antibodies. Large arrow, male pronucleus; small arrows, female pronuclei. Bar, 10  $\mu$ m.

jected histones could assemble onto sperm chromatin and induce its condensation in the absence of protein synthesis. The injected histones became associated with the chromatin, but the chromatin remained in a dispersed state. This suggests that, although histones may be synthesized during meiotic maturation, other proteins synthesized during maturation are required for transition from the dispersed to the condensed state. Assuming that condensation reflects the assembly of somatic-like chromatin that is able to respond to its cytoplasmic environment, it may be inferred that these proteins are involved in chromatin assembly or chromatin structure.

Numerous changes in the pattern of protein synthesis occur during maturation (Schultz and Wassarman, 1977a,b), yet, except for histones and several regulators of the cell cycle (Picard *et al.*, 1985; Hashimoto and Kishimoto, 1988; O'Keefe *et al.*, 1989, 1991; van de Woude *et al.*, 1990; Colas *et al.*, 1993; Gavin *et al.*, 1994), the majority of products remain unidentified. In the frog oocyte, nucleoplasmin and nuclear protein N1 remove protamines and transfer histones onto sperm chromatin and are synthesized during oogenesis (Kleinschmidt *et al.*, 1985, 1990; Dilworth *et al.*, 1987; Philpott *et al.*, 1991). It is possible that homologous proteins are synthesized by the mouse oocyte during the early stages of maturation. If this is the case, it may be that in the absence of protein synthesis, the microinjected histones are not properly transferred onto the sperm DNA

such that an abnormal nucleoprotein complex is assembled. Alternatively, these newly synthesized proteins may participate in another aspect of chromatin assembly.

### **Functional Sperm-Derived Chromatin Produced after Residency in Maturing Oocyte Cytoplasm**

Several considerations suggest that the remodeling of the sperm nucleus within maturing oocyte cytoplasm produces functional somatic-like chromatin. First, following normal fertilization at metaphase II, the sperm chromatin becomes dispersed and briefly condensed, before decondensation within the male pronucleus, and this is temporally correlated with protamine-histone exchange (Ecklund and Levine, 1975; Wright and Longo, 1988). Thus, sperm nuclei introduced into maturing oocytes and into activated eggs undergo similar biochemical and morphological changes. Second, sperm-derived chromatin within maturing oocyte cytoplasm forms chromosome-like bodies (Figs. 3C and 3D; Clarke and Masui, 1986) that become associated with a spindle (Harrouk and Clarke, 1993). Third, following parthenogenetic activation of the inseminated oocytes, the sperm-derived chromatin forms a pronucleus which together with the maternally derived pronucleus undergoes DNA replication. Thus, sperm chromatin that has been remodeled in maturing oocyte cytoplasm behaves as a normal male pronucleus following activation. The functional competence of the remodeled sperm chromatin strongly implies that the histones and other DNA-binding proteins have been properly assembled onto the sperm DNA.

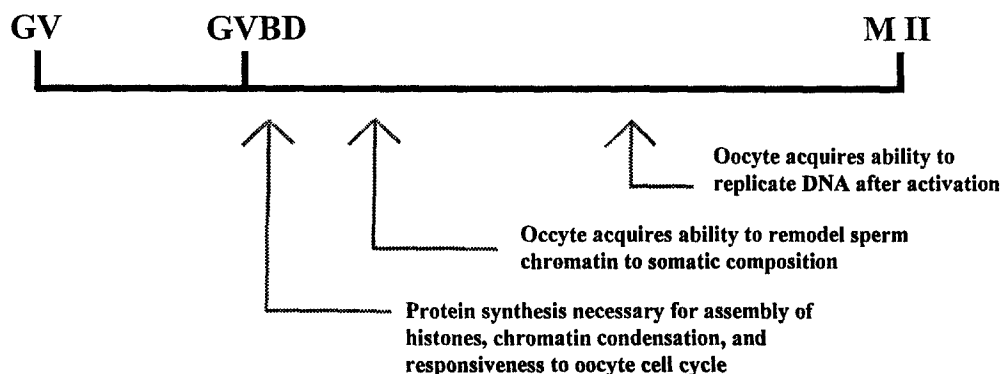
These results together suggest that the mechanisms that control the reorganization of the sperm nucleus into somatic-like chromatin, which in mice normally operate following fertilization at metaphase II, are present and active in maturing, unactivated oocytes. In several invertebrate organisms, such as the surf clam (*Spisula solidissima*), sperm normally fertilize the oocyte at the GV stage and trigger meiotic maturation (Longo and Anderson, 1970a,b; Chen and Longo, 1983; Luttmner and Longo, 1988). In this case, the maturing oocyte cytoplasm presumably is capable

**TABLE 3**

Dependence of BrdU Incorporation into Male and Female Pronuclei on Duration of Remodeling before Parthenogenetic Activation

Duration of incubation after insemination (hr)	Number of eggs examined	BrdU staining <sup>a</sup>	
		–	+
11	20	20	0
17	51	11	40

<sup>a</sup> Inseminated oocytes were left to mature for 11 or 17 hr before activation. Following activation oocytes were incubated 18 hr in 0.4 mM BrdU, fixed, and analyzed by immunocytochemistry.



**FIG. 7.** Summary of chromatin remodeling capabilities gained during oocyte maturation. With the onset of meiotic maturation the protamine to histone transition can be completed with the resulting remodeled chromatin being under the control of the oocyte cell cycle. Later the oocyte develops the ability to synthesize DNA following fertilization and pronuclear formation.

of remodeling the sperm chromatin to form embryonic chromatin. In the frog, sperm nuclei form metaphase chromosomes when incubated in extracts prepared from unactivated oocytes at metaphase II (Lohka and Masui, 1983; Ohsumi *et al.*, 1993), which suggests that the capacity of the frog oocyte to remodel sperm chromatin does not depend on activation. Thus, it may be generally true that the oocyte mechanisms that remodel sperm nuclei into somatic-like chromatin are present or develop during meiotic maturation. Since these mechanisms, which in vertebrates normally operate in activated eggs where MPF activity is low, are apparently active in maturing oocytes, where MPF activity is high, it may be inferred that they are not regulated by cell-cycle dependent activities.

Our experiments also showed that the capacity to undergo DNA replication develops during maturation. When oocytes were incubated for 11 hr after insemination and then parthenogenetically activated, both oocyte and sperm chromatin formed pronuclei but neither could subsequently synthesize DNA. When the incubation time was extended to 17 hr prior to activation, both pronuclei underwent DNA replication. This clearly shows that the ability to initiate DNA replication develops late during maturation and is separate from the ability of the oocyte to be parthenogenetically activated as judged by pronuclear formation. As maturing mouse oocytes are capable of DNA repair (Brazill and Masui, 1978), the maturation-dependent event apparently pertains specifically to DNA replication. Similar results have been reported in the frog, where the ability to initiate DNA replication is acquired during maturation, possibly as a result of decline in the level of inhibitors present in prophase oocytes (Zhao and Benbow, 1994).

In summary, our results show that the ability to restructure the nonnucleosomal, protamine-associated DNA of the sperm into functional somatic-like chromatin develops in oocytes during meiotic maturation, requires proteins synthesized during maturation, and can be expressed independently of activation (Fig. 7). It will be interesting to examine

whether partially grown, meiotically incompetent oocytes, which can be induced to undergo GVBD by exposure to okadaic acid, possess the ability to remodel sperm chromatin properly. The results will identify the stages of oocyte growth when the genes encoding the necessary factors, such as histones and the factors mediating their assembly onto sperm chromatin, are expressed. In addition, it will be important to determine the genomic imprinting pattern of the sperm DNA that has become combined with oocyte chromosomal proteins within the maturing oocyte cytoplasm. This will help to identify the relationships between DNA and protein during the chromatin imprinting process.

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